PRACTICAL FLOW CYTOMETRY

Fourth Edition

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and because the energy must be passed through secret Swiss bank accounts. The associated emission is called phosphorescence. Lifetimes for phosphorescence are much longer than for fluorescence, typically milliseconds to seconds, thanks to which we have television and watch dials that glow in the dark. Since the energy difference between T_1 and the ground state S_0 is usually smaller than the energy difference between S_1 and S_0, phosphorescence typically occurs at longer wavelengths than fluorescence.

Fluorescence Polarization

I have already mentioned that, in order for absorption to occur, the E vector of the incident light must be aligned with the dipole moment of the absorbing molecule. If the incident light is linearly polarized, only those molecules that happen to be oriented properly with respect to the plane of polarization will absorb light. Since only those molecules absorb the incident light, only they are capable of fluorescence emission. Like absorption, emission can only occur in a direction determined by the orientation of the molecule.

Absorption occurs so rapidly (10^{-15} s) that the absorbing molecules have no time to move during the process. If fluorescence emission occurred as rapidly as absorption, or if the molecules involved were completely immobilized, the fluorescence emission occurring following excitation by linearly polarized light would be linearly polarized, although not necessarily polarized in the same plane as the exciting light. By now, we know that that isn't the way the world works; fluorescence is going to occur over a period of nanoseconds following absorption, and it's a cinch that at least some of the molecules are going to change their orientations (i.e., rotate) before they emit. This means that some fluorescence depolarization will occur. The more motion there is before emission, the more depolarization we can expect.

We can make use of this effect to determine the relative rotational freedom of fluorescent molecules, or the fluidity of their microenvironment; or, looking at the other side of the coin, we can determine the extent to which molecular movement is restricted, or the viscosity of the microenvironment. This is done by using appropriate polarization optics, which may be as simple as Polaroid filters in different orientations, and making measurements of fluorescence intensities polarized in the planes parallel and perpendicular to the plane of polarization of the excitation. These intensities are, respectively, denoted by $I_\parallel$ and $I_\perp$.

From the intensities, we can compute either the fluorescence polarization, p, as

$$p = (I_\parallel - I_\perp)/(I_\parallel + I_\perp),$$

or the fluorescence emission anisotropy, r, where

$$r = (I_\parallel - I_\perp)/(I_\parallel + 2\,I_\perp).$$

Values of both polarization and anisotropy increase as molecular rotation is increasingly restricted. The use of fluorescence polarization and anisotropy measurements to measure rotational diffusion of molecules in membranes and the cytosol will be discussed further in the chapter on parameters and probes.

As a general rule, when we are not trying to measure anisotropy or polarization in our cytometers, we pay little or no attention to the degree or direction of polarization of fluorescence. Most of the time, we get away with it. However, an article published in 2000 by Asbury, Uy, and van den Engh[243] suggests that polarization effects may represent a fair-sized skeleton in our cytometric closet.

Since the light emitted by most lasers used as light sources in cytometry is polarized, both scattered light and fluorescence emission are typically polarized to some degree. This makes the intensity of detected signals more dependent on the angle and direction at which they are detected than would otherwise be the case. Differences from instrument to instrument in optical geometry, and in the polarization response of optical elements such as lenses, dichroics, and filters, may therefore lead to otherwise inexplicable differences in the intensities of signals measured from supposedly identical cells or particles. Further complications may be introduced by the fact that different fluorescent probes exhibit differing degrees of fluorescence polarization, some intrinsic to the molecular structure of the probes, and some dependent on binding to macromolecules and on other environmental characteristics.

The bottom line for most users is that polarization-related differences in the response of different instruments may interfere with the standardization of quantitative fluorescence measurement. The bottom line for those of us who develop and manufacture instruments is that we need to determine the nature and extent of those differences, in hopes of reconciling results from existing systems and improving the design of future systems. A simple solution was suggested by Asbury, Uy, and van den Engh; placing a polarizer at the so-called "magic angle" (54.7° for linearly polarized source emission) in the light path of each fluorescence detector removes the dependence of intensity measurements on polarization, with only a modest loss of overall signal intensity.

Stimulated Emission

One of the stranger things photons can do is make more photons just like themselves. I don't mean from nothing; there has to be some energy input to start with, but it's still pretty remarkable. It took Einstein to figure it out. We already know that a photon is likely to be absorbed by a molecule if the energy difference between the molecule's ground and excited states is equal to the energy of the photon. It turns out that just having photons of that energy around also increases the likelihood that molecules already excited will emit identical photons.

That, of course, is the catch. In general, when we're talking electronic excitation, there are a lot fewer excited molecules than molecules in the ground state, as our exercises